ABSTRACT:

Percutaneous absorption of pesticides is a major determinant for risk assessment. Furthermore, cutaneous metabolism plays a role in penetration of certain chemicals. Therefore, the aim of these studies was to determine the transdermal metabolism of three related compounds [the herbicide, fluroxypyr methylheptyl ester (FPMH), fluroxypyr methyl ester (FPM), and fluroxypyr (FP)] during penetration through human and rat skin in vitro. The data presented in this article show that both FPM and FPMH were completely metabolized during their passage through human and rat skin in vitro. The only metabolite produced was that of the hydrolysis product, FP, with no parent ester penetrating through the skin. The extent of FP formation within the skin was directly correlated to the degree of stratum corneum reservoir formation. The larger the stratum corneum reservoir, the lower the levels of FP recovered from within the skin. This suggests that as the ester partitioned out of the SC it was immediately hydrolyzed to FP, which could then pass freely through the remainder of the epidermis and dermis. Similar metabolic profiles were observed for the transdermal metabolism of FPM and FPMH in previously frozen rat skin, indicating the robust nature of the esterase enzymes involved. In conclusion, systemic exposure after skin contact with FPM or FPMH is likely to be to the acid metabolite, FP, only and not to the parent ester. In addition, the rate and extent of percutaneous absorption will be a major determinant of cutaneous metabolism.

The skin is the predominant route of exposure to agrochemicals, which are absorbed through both human and rat skin in vitro (Hewitt, 1996). Chemicals, if deposited on the skin surface, can be absorbed into the systemic circulation. However, xenobiotic metabolizing enzymes are present in the skin, indicating the potential for topically applied compounds to undergo substantial cutaneous metabolism during penetration (Hotchkiss, 1992; Ademola et al., 1993; van de Sandt et al., 1993). The skin is able to carry out a wide range of Phase I and II metabolic biotransformations. The specific activities of each enzyme system in the skin are much lower than their hepatic counterparts; however, taking into account the large surface area and volume of the skin, the dermal metabolism can be extensive. The highest levels of these enzymes are found within the hair follicles and sebaceous glands, as well as the epidermis (Bickers et al., 1982; Finnen and Shuster, 1985; Finnen, 1987; Mukhtar et al., 1987).

Not only does cutaneous metabolism introduce new chemicals into the systemic circulation, it can also be a very important determinant in the penetration of certain compounds through the skin, as is the case for benzo[a]pyrene (Smith and Holland, 1981). Polar metabolites will be better able to penetrate the aqueous environment of the dermis and epidermis. The necessity for skin viability for in vitro percutaneous absorption studies is therefore paramount. Initial studies carried out in our laboratory by Garnett (1992) showed that the viability of human and rat skin in this in vitro skin absorption model was maintained up to 24 h.

This article describes in vitro experiments conducted to determine whether the “first-pass” metabolism of two herbicides occurs during penetration through and into human and rat skin. Fluroxypyr methylheptyl ester [1-methylheptyl-4-amino-3,5-dichloro-6-fluoro-2-pyridyloxycetic acid (FPMH)]; Fig. 1, which is marketed as Starane (Dow AgroSciences, Indianapolis, IN)]. FPMH is manufactured from the intermediate fluroxypyr methyl ester (FPM; Fig. 1), and is rapidly hydrolyzed within the target plant to form fluroxypyr (FP; Fig. 1). The metabolism of FPM has not been previously studied in either humans or animals. However, much work has concentrated on the breakdown of FPMH in plants, soil, water, and rats. FPMH is hydrolyzed (nonenzymatically) to FP, as well as other degradation products (Lehmann et al., 1990, 1993). Administration of FP to rats has shown no additional metabolism of the acid, but complete and rapid excretion of unchanged FP in the urine (Dow AgroSciences, unpublished data).

The effect of freezing the skin on cutaneous absorption and metabolism was also studied. The studies were carried out using full-thickness skin in flow-through diffusion cells, using an in vitro tech-
skin was in contact with a continuously flowing receptor fluid of HEPES-buffered Hank’s balanced salt solution (HBSS) supplemented with 0.5% (v/v) gentamicin (degassed before use by filtration through a 0.5-μm filter) at a flow rate of 1.5 ml/h. The skin was maintained at a constant temperature of 32.0 ± 1.0°C.

**Dose Application and Determination of Absorption.** Aliquots (20 μl) of [ring-14C]-FP (2352 μg/cm² in ethanol), [ring-14C]-FPM [1382 μg/cm² in ethanol (human) and 676 μg/cm² (rat)], or [ring-14C]-FPMH [1413 μg/cm² in ethanol; as the EC (Starane 250; 7.8 mg/cm²)] were applied to the epidermis (outer layer) of the skin (0.32 cm²) using a blunt-ended microsyringe, and the skin was occluded with a Teflon screw cap, which, when completely tightened, lies 1.9 cm above the surface of the skin. The receptor fluid (3 ml) was collected every 2 h for 48 h into 20-ml glass scintillation vials and 3 × 100-μl aliquots were taken to determine the amount of radioactivity present (by liquid scintillation spectrometry (LSS); Packard Tri-Carb model 4640). Twenty microliters of the remaining receptor fluid was injected directly onto the HPLC column, for the elution of the parent compounds and possible metabolites.

**Determination of Residual Radioactivity. Measurement of residual radioactivity remaining on the skin surface.** At the end of each experiment, the skin was washed with an aqueous liquid hand soap solution (2% v/v) as previously described (Hewitt, 1996). Aliquots of these washes were also taken for HPLC analysis, to determine any degradation on the skin surface.

**Metabolite Determination. Sample preparation.** Perfusate (receptor fluid that had bathed the underside of the skin) and ethanolic solutions containing the skin surface, cell, and Teflon cap washes were injected directly onto the HPLC column and analyzed for the presence of metabolites. The stratum corneum (SC) was removed by tape stripping the cleaned skin 12 times with conventional cellophane tape and placing the tape into ethanol overnight. These ethanol extracts were analyzed by HPLC. The remainder of the viable skin was homogenized by cutting the circle of skin into small pieces and adding 5 ml of cold HPLC mobile phase [acetonitrile/water (70:30 v/v)]. The skin was homogenized by three 10-s bursts from the probe of an Ultra-turrax homogenizer (T25; IKA-Werke GmbH, Staufen, Germany). This crude homogenate was centrifuged at 2200g for 20 min, and the supernatant passed through a 0.45-μm solid-phase syringe filter (Whatman, Clifton, NJ). This extract was then analyzed for metabolites by HPLC.

**Determination of parent compounds and possible metabolites.** The metabolites and parent compounds were identified by the use of a reverse-phase HPLC assay using UV detection at 226 nm. An ODS-2 RP18 HPLC column was used (250 × 5-mm i.d.) (Merck KGaA, Darmstadt, Germany), which was fitted with an RP18 precolumn (Merck). The compounds were eluted using an isocratic flow of a filtered mobile phase (0.45-μm filter under pressure), which was either acetonitrile/water (38:62 v/v; pH = 2.75) for the detection of FP and FPM, or acetonitrile/water (70:30 v/v; pH = 2.75) for the detection of FPMH. Analysis was carried out at room temperature at a flow rate of 1.5 ml/min. Twenty-microliter injections of each sample were eluted and metabolites and/or parent compounds were identified by comparing retention times with those of authentic standards. The presence of the parent compound and the acid metabolite were confirmed by collecting 18-s fractions of eluent after injection of radiolabeled samples and standards; these fractions were then assayed by LSS. FP standard solutions gave a retention time of 4.2 min, and a mean standard curve gave a correlation coefficient (r²) of 0.995; whereas the limit of detection for this HPLC method was 5 ng/injection (equivalent to 0.5 μg/ml). FPM retention time was 11.4 min, and a mean standard curve gave an r² of 1.00, whereas the limit of detection was 5 ng/injection (equivalent to 0.5 μg/ml). FPMH eluted at 8.5 min, with a mean standard curve of peak area versus FPMH concentration giving an r² of 1.00, and a limit of detection of 0.1 ng/injection (equivalent to 0.01 μg/ml).

**Statistical Evaluation.** The unpaired Student’s t test was used for the comparison of fresh and frozen skin data. The level of significance was taken as P < .05.
and rat skin at 48 h are shown in Table 1. Only the presence of the acid detected in the viable skin homogenates, skin surface washes, or cell showed only the presence of the applied compound. No FP or any Table 1. HPLC analysis of these receptor fluid samples (0 – 48 h) for FP through full-thickness human and rat skin at 48 h are shown in
underside of the skin for 24 h was slightly higher, although still less 2%. The degree of hydrolysis to FP in HHBSS exposed to the
ester (FPM), with no hydrolysis product, FP, detected (Fig. 2). Sim-
ilarly, analysis (HPLC and LSS) of rat SC at 48 h showed the presence of FPM and FPMH to FP occurred in HHBSS, but this was less than
bSkin samples were not tape-stripped.

Results

Control Incubations. A small amount of nonenzymatic hydrolysis of FPM and FPMH to FP occurred in HHBSS, but this was less than 2%. The degree of hydrolysis to FP in HHBSS exposed to the underside of the skin for 24 h was slightly higher, although still less than 5%.

Transdermal Metabolism. FP. The percutaneous absorption data for FP through full-thickness human and rat skin at 48 h are shown in Table 1. HPLC analysis of these receptor fluid samples (0–48 h) showed only the presence of the applied compound. No FP or any other metabolites (either UV or radiolabeled) were detectable at any time in either human or rat skin. Similarly, no metabolites of FP were detected in the viable skin homogenates, skin surface washes, or cell body/nut washes, for either human and rat skin (data not shown).

FPM. The percutaneous absorption data for FPM through human and rat skin at 48 h are shown in Table 1. Only the presence of the acid metabolite, FP, was detected in the receptor fluid. This amounted to a total of 45.3 ± 7.1 and 51.6 ± 8.0 µg/cm² after 48 h for human and rat skin, respectively (Fig. 2). No parent compound (FPM) was detected in the viable skin or receptor fluid at any time. SC after 48 h (which was separated from the viable skin by tape stripping) showed the presence of one major UV peak, identified as FPM [66.1 ± 9.0% of the total skin residue (SC + viable) (187.9 ± 25.4 µg/cm²)]. In contrast, the metabolite, FP, was the only compound detected in the viable skin [33.9 ± 5.0% of the total material recovered; 14.4% of the total material recovered; (96.2 ± 14.3 µg/cm²)] (Fig. 2). Liquid scintillation counting of the HPLC eluents showed the presence of no other radioactive peaks. SC contained one radioactive peak corresponding to the [14C]-FPM standard and viable skin contained one radioactive peak corresponding to that of [14C]-FP. Material recovered from the skin surface and Teflon cell body wash was also entirely the parent ester (FP), with no hydrolysis product, FP, detected (Fig. 2). Similarly, analysis (HPLC and LSS) of rat SC at 48 h showed the presence of FPM only [55.2 ± 11.1% (167.1 ± 33.5 µg/cm²)], whereas the viable skin contained only FP [44.8 ± 5.3% (n = 3) (135.4 ± 16.0 µg/cm²)] and (Fig. 2).

FPMH (Ethanol). The percutaneous absorption of FPMH through human and rat skin in vitro at 48 h is shown in Table 1. The receptor fluid (0–48 h) contained only the acid metabolite, FP, which amounted to a total of 6.6 ± 1.1 µg/cm² (human) and 72.2 ± 12.9 µg/cm² (rat) after 48 h (Fig. 3). The distribution of parent compound and FP reflected that observed for FPM. SC contained only the parent compound, FPMH, (68.9 ± 14.4% of the total material recovered; 40.7 ± 8.5 µg/cm²) and the viable tissue contained only the metabolite (FP) (31.1 ± 4.5% (n = 3) of the total material recovered; 18.4 ± 2.7 µg/cm²). No other radioactive peaks were present in either the SC or viable tissue. Again, skin surface and cell washes contained only parent ester (Fig. 3). SC from rat skin contained only FPMH (81.4 ± 9.2% of the total material recovered; 478.4 ± 53.8 µg/cm²) and the viable skin contained only FP (18.6 ± 2.1% of the total material recovered; 109.6 ± 12.5 µg/cm²) (Fig. 3). Only FPMH was detected on the skin surface and on the cell body.

FPMH (EC). The percutaneous absorption of the formulated product, FPMH (EC), through human and rat skin in vitro at 48 h is shown in Table 1. The metabolite distribution profile of FPMH (EC) was very similar to that seen for the unformulated product. Only the parent compound was found on the skin surface and within the SC, whereas only the metabolite, FP, was recovered from the viable skin and the receptor fluid (Fig. 4). The absolute amounts recovered were much higher for FPMH (EC), because a 5-fold greater dose was applied to the skin.

Transdermal Metabolism in Previously Frozen Rat Skin In Vitro. Absorption of both esters through previously frozen rat skin was significantly increased (Table 2). However, the metabolite distribution within the skin was unaffected by freezing.

Discussion

The data presented herein show that both FPM and FPMH (applied in either an ethanol vehicle or as the EC formulation) were completely metabolized during their passage through human and rat skin in vitro. Hydrolysis occurred only in the viable layers of the skin, but remained unchanged in the SC reservoir. The only metabolite produced was the hydrolysis product, FP, with no additional metabolites being detected. This is in agreement with in vivo metabolism in rats, whereby only FP was excreted in the urine and feces after i.v. administration of FPMH (Dow AgroSciences, unpublished data). This has also been demonstrated for the herbicide, fluazifop butyl, where 96% of the skin-extractable material was located in the SC, as the parent ester. As the ester passed from the SC into the viable skin beneath, metabolism to fluazifop acid was extremely rapid (Clark, 1993).

The degree of metabolism within the skin was species-dependent as well as compound-specific. The greater extent of metabolism observed in rat skin may be due to extensive absorption into the viable layers compared with human skin (Hewitt, 1996). Therefore, more of the applied dose is likely to come into contact with the skin esterases responsible for its metabolism. In addition, there are differences in the metabolic activities of human and rat skin. High drug metabolizing activities have been associated with the differentiated cells of the hair follicles and adjacent sebaceous glands (Wilton Coomes et al., 1983; Hukkelhoven et al., 1984; Kao et al., 1988). The rat has a much higher number of hair follicles per unit area of skin; therefore, the metabolic capability of rat skin may be greater than human skin. The reservoir capacity of rat skin was much greater than human skin in that a higher percentage of the parent compound remained in the SC of the rat than that of the human skin. Other factors may also influence dermal metabolism. For example, FPMH is highly lipophilic (log P'ow = 4.53), which causes it to partition very well into the SC. Therefore, the majority of FPMH remains on the skin surface and in the SC, even after 48 h (Hewitt, 1996) and less compound is available for hydrolysis to FP in the viable layers. HPLC analysis of the SC showed no esterase hydrolysis, thus, the skin reservoir was entirely comprised of parent compound.
Application of FPMH as an EC marginally increased the ester hydrolysis to FP in the rat (Figs. 3 and 4). This increase in metabolism correlates with a decreased SC reservoir capacity for FPMH (EC) (66%) compared with the unformulated compound (81%). Therefore, the formulation itself appears to affect the barrier properties of the SC, leading to greater penetration into viable skin and increased metabolite production.

There have been a number of reports demonstrating the presence of both phase I and phase II metabolizing systems in rat and human skin (Pannatier et al., 1978; Kao and Carver, 1990; Hotchkiss, 1992; Pannatier et al., 1978; Kao and Carver, 1990; Hotchkiss, 1992;
Ademola et al., 1993; van de Sandt et al., 1993). Cutaneous esterases have not been isolated and characterized to date, although the skin is capable of considerable nonspecific esterase activity, metabolizing many different topically applied drugs (Meyer and Neurand, 1976; Guzek et al., 1989; Mint, 1995). Therefore, it is becoming increasingly evident that percutaneous absorption involves metabolic as well as diffusional processes. The relative importance of these processes in skin penetration will be dependent on the physicochemical properties of the compound and its metabolites. For the highly lipophilic compounds, such as FPMH, the SC is not the diffusion barrier, because

Fig. 3. Relative amounts of FPMH and the metabolite FP from the skin surface, SC, viable skin, and receptor fluid after application of FPMH to human (□) and rat (■) skin in vitro, mean ± S.D., n = 3 separate donor skins.
this compound rapidly enters this layer. The rate-limiting step is the diffusion into the layers beneath it. Once in the viable layers, the rate of metabolism is very rapid (as predicted by the kinetic data; Hewitt et al., 2000), producing the very hydrophilic molecule, FP, which can readily pass through the viable tissue into the receptor fluid. Conversely, the absorption of highly hydrophilic compounds such as FP through full-thickness skin is low because the lipid-rich SC presents a diffusion barrier. However, when the SC is removed (by tape-strip-
and the total recoveries were both 90%. Skins from the same animal as the fresh skin. The same dose was applied to fresh and frozen skins, respectively (Hewitt, 1996).

It has been suggested that metabolism observed during in vitro studies may be a result of compound decomposition on the skin surface (Bungaard and Hansen, 1981) or hydrolysis in the receptor fluid (Kao, 1990). However, this was not the case here, because control incubations of FPM and FPMH in HHISS showed only minimal hydrolysis to FP (≤2%). Analysis of the skin surface washes showed no breakdown of the esters, or FP itself, before penetration into the skin. Yu et al. (1980) also proposed that enzymes that leaked from the skin cells could leach out of the tissue into the receptor fluid and therefore any parent compound absorbed through the skin would be hydrolyzed in the receptor fluid, thus attenuating the extent of cutaneous metabolism. This was also ruled out as a factor in these studies because when the two esters were incubated with HHISS, which had been collected after passing beneath untreated human and rat skin for 24 h, less than 5% hydrolysis to FP was observed.

Freezing rat skin at −20°C for up to 6 weeks had no effect on the activity of the skin esterases involved in the metabolism of FPM and FPMH, although overall penetration through the skin was significantly increased. Various workers have reported the effects of freezing skin on percutaneous absorption (Swarbrick et al., 1982; Hawkins and Reifenrath, 1984; Bronaugh et al., 1986), but little has been published on the metabolic capability of frozen skin. Garnett (1992) reported that previously frozen human and rat skin showed substantial esterase activity toward benzylic acetate. Taken together, these findings suggest that frozen skin may be used in transdermal metabolism studies where esterases are involved. However, the stability of other cutaneous enzyme systems, such as cytochrome P450, has yet to be evaluated.

In conclusion, both human and rat skin are able to fully metabolize FPM and FPMH, but only after they have partitioned out of the SC into the viable, esterase-rich environment of the epidermis. The data presented here indicate that systemic exposure after skin contact with FPM and FPMH is likely to be to the acid metabolite, FP, only and not the parent ester. It is clear that the rate and extent of percutaneous absorption into the skin and the formation of a reservoir in the SC will affect the degree of cutaneous metabolism. However, the rate of metabolism of a compound, once it has reached the SC/viable tissue interface, is also an important factor affecting its percutaneous absorption.

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### References


